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Evaluation of immunophenotypic and molecular biomarkers for Sézary syndrome using standard operating procedures: *multicenter study of 59 cases*

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Abbreviations:

SS = Sézary syndrome

CTCL = cutaneous T cell lymphoma

EID = erythrodermic inflammatory dermatoses

EORTC = European Organization for Research and Treatment of Cancer

CNV = copy number variation

GE = gene expression

PBMC = peripheral blood mononuclear cell

SOP = standard operating procedures

HC = healthy controls

ABSTRACT

Differentiation between Sézary syndrome (SS) and erythrodermic inflammatory dermatoses (EID) can be challenging and a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could be useful as additional diagnostic criterion. In this European multicenter study the sensitivity and specificity of these immunophenotypic and recently proposed but unconfirmed molecular biomarkers in SS was investigated. Peripheral blood CD4⁺ T-cells from 59 SS and 19 EID patients were analyzed for cell surface proteins by flow cytometry, and for copy number alterations and differential gene expression using custom made qPCR plates. Experiments were performed in duplicate in two independent centers using standard operating procedures with almost identical results. Sézary cells showed *MYC* gain (40%) and *MNT* loss (66%), upregulation of *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%) and *PLS3* (66%) and downregulation of *STAT4* (91%). Loss of CD26 ($\geq 80\%$ CD4⁺ T-cells) and/ or CD7 ($\geq 40\%$ CD4⁺ T-cells) and combination of altered expression of *STAT4*, *TWIST1* and *DNM3* or *PLS3*, could distinguish respectively 83% and 98% of SS patients from EID cases with 100% specificity. These additional diagnostic panels will be useful adjuncts in the differential diagnosis of SS versus EID.

INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T cell lymphoma (CTCL) derived from CD4⁺ skin-homing memory T-cells characterized by erythroderma, generalized lymphadenopathy and neoplastic T-cells (Sézary cells) in skin, lymph nodes and peripheral blood (Wieselthier and Koh, 1990).

Differentiation between SS and erythrodermic inflammatory dermatoses (EID) may be extremely difficult, both clinically and histopathologically, but is very important from therapeutic and prognostic perspective. For a long time the diagnosis was based on demonstration of atypical T-cells, so called Sézary cells, in blood smears (Sentis et al., 1986; Trotter et al., 1997). However it was demonstrated that Sézary cells can also be observed in the peripheral blood of patients with EID and even in normal controls (Meijer et al., 1977; Duncan and Winkelmann, 1978). Demonstration of at least 1000 Sézary cells per mm³ was often used as a decisive criterion, but was not generally agreed upon (Wieselthier and Koh, 1990). To prevent that patients with EID would be misclassified as SS and treated as such, in 1997 the European Organization for Research and Treatment of Cancer (EORTC) group proposed demonstration of clonal T-cells and presence of an expanded CD4⁺ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS (Willemze et al., 1997).

At present the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in the skin), in combination with one or more of the following criteria: an absolute Sézary cell count > 1000 cells per mm³; loss of T-cell markers CD2, CD3, CD4 and /or CD5 and /or an expanding population of CD4⁺ T-cells leading to a CD4/CD8 ratio of more than 10 (Willemze et al., 2005; Swerdlow et al., 2008). However, distinction between SS and EID can still be difficult as T-cell clonality can be observed in a substantial proportion of EID as well,

and not all SS patients have a CD4/CD8 ratio >10 at first presentation (Vonderheid, 2006; Vonderheid and Bernengo, 2003).

To solve this diagnostic problem a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells. Flow cytometry studies reported loss of CD7 and CD26 expression by Sézary cells and suggested CD4+CD7– cells of at least 40% and CD4+CD26– cells of 30% or more as tentative diagnostic criteria in those difficult cases (Harmon et al., 1996; Rappl et al., 2001; Washington et al., 2002; Lima et al., 2003; Sokolowska-Wojdylo et al., 2005; Klemke et al., 2008; Fierro et al., 2008; Bernengo et al., 2001; Jones et al., 2001; Kelemen et al., 2008; Vonderheid et al., 2002; Vonderheid and Bernengo, 2003; Olsen et al., 2007).

In addition, recent studies described expression of killer cell immunoglobulin (KIR)-like receptors CD158a, CD158b, CD158k and the “central memory” T-cell phenotype (CD27+, CD45RA–, CD45RO+) as characteristic features of Sézary cells (Bagot et al., 2001; Marie-Cardine et al., 2007; Poszepczynska-Guigne et al., 2004; Bahler et al., 2008; Klemke et al., 2008; Michel et al., 2013; Lima et al., 2003; Fierro et al., 2008; Dummer et al., 1996; Karenko et al., 2001; Campbell et al., 2010). Molecular investigations identified gain of *JUNB*, *MYC* and loss of *MYC* antagonists *MNT* and *MXI1* as recurrent genetic lesions in the SS genome (Mao et al., 2003; Mao et al., 2008; Vermeer et al., 2008). Gene expression studies demonstrated increased expression of *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *JUNB*, *TWIST1*, *EPHA4* and *MYC* and decreased expression of *STAT4* in Sézary cells (Su et al., 2003; Kari et al., 2003; Nebozhyn et al., 2006; Booken et al., 2008; Mao et al., 2008; Van Doorn R. et al., 2004; Goswami et al., 2012).

However, the diagnostic value of these biomarkers in diagnosing SS has not been investigated thoroughly. Moreover, most biomarkers were identified in small, single center studies with limited number of patients and controls and have not been confirmed in large independent

studies. In addition, flow cytometry studies have used widely differing protocols that impede interpretation and comparison of results from different studies.

The goal of this EORTC multicenter study is to investigate the sensitivity and specificity of these biomarkers for SS in a large group of well-defined SS patients compared to EID patients using standard operating procedures.

RESULTS

Patient characteristics

Clinical characteristics at diagnosis of the 59 SS patients and 19 EID patients are summarized in **Table 1**.

All Sézary patients had a T-cell clone in the peripheral blood (59 of 59), a CD4/CD8 ratio above 10 (53 of 57) and/or a Sézary cell count above 1000 mm³ (34 of 43), including all four patients with a CD4/CD8 ratio lower than ten.

One EID patient showed a T-cell clone in the peripheral blood, another EID patient had a CD4/CD8 ratio above 10 due to very low numbers of CD8+ T-cells, but none had a Sézary cell count above 1000 mm³ (**Table 1**).

Flow cytometry

Flow cytometry experiments were performed both in Leiden and Paris for all 59 SS patients and 19 EID patients. Differences in flow cytometry results between Leiden and Paris was < 20% in 99.8% of individual assays and in these cases an average was used in further analysis.

In only two of 1027 assays (0.2%) the differences in results exceeded 20% and these were therefore excluded from further analysis.

In this study, 87% of the SS patients (46 of 53) had a CD4/CD8 ratio above 10 at inclusion, compared to 8% of the EID patients (1 of 12) (sensitivity: 87%; specificity: 92%).

The CD4⁺ gated lymphocytes were CD3⁺ and CD8[−]. In the CD4⁺ T-cell population seven of 59 (12%) SS patients showed loss for CD2 (median 45%; range 32-100%) and four (7%) patients showed diminished expression for CD2 (CD2^{dim}), while this was never observed in the 19 EID cases. One Sézary patient showed 90% CD5 loss, compared to none of the EID cases.

In the CD4⁺ T-cell population a percentage of CD4⁺CD7[−] cells above 40% was found in 32 of 59 (54%) Sézary patients but never in EID patients (sensitivity: 54%; specificity: 100%). In addition, CD7^{dim} was found in 2 of 59 (3%) SS cases, compared to none of the EID patients.

In the CD4⁺ T-cell population a percentage of 30% or more CD4⁺CD26[−] cells was found in 51 of 59 (86%) Sézary patients but also in 10 of 19 (53%) EID patients (sensitivity: 86%; specificity: 47%). When shifting the percentage to 80%, 39 of 59 (66%) SS patients but none of the EID patients had CD4⁺CD26[−] cells of 80% or more in the CD4⁺ T-cell population (sensitivity: 66%; specificity: 100%). In addition, CD26^{dim} was found in 5 of 59 (8%) Sézary patients and in one of 19 (5%) EID patients.

Loss of CD26 by more than 80% and/ or loss of CD7 by more than 40% of CD4⁺ T-cells was found in 49 of 59 (83%) Sézary patients but was never observed in the EID patients (sensitivity: 83%; specificity: 100%).

Investigations on CD158k expression showed that more than 5% of these CD4⁺ T-cells expressed CD158k or CD158k^{low} in 19 of 58 (33%) SS patients, compared to one of 19 (5%)

EID cases (sensitivity: 33%; specificity: 95%). The results (including expression of CD158a and CD158b) are summarized in **Table 2**.

No major difference was observed in the expression of CD27, CD45RA and CD45RO by CD4+ T-cells from SS and EID patients (data not shown).

Copy number variation

CNV experiments were performed in 58 SS patients and 17 EID patients in Leiden. Duplicate experiments for 14 samples were performed in London which gave identical results (**Figure S1**).

In 47 of 58 (81%) SS cases alterations in copy number were found compared to none of the 17 EID patients. Gain of *MYC* was observed in 23 of 58 (40%) SS patients (sensitivity: 40%; specificity 100%). *MNT* loss was found in 38 of 58 (66%) SS patients (sensitivity: 66%; specificity: 100%) and one (2%) patient showed gain of *MNT* (**Figure 1**). Gain of *MYC* and/or loss of *MNT* was found in 76% (44 of 58) of SS cases (sensitivity: 76%; specificity: 100%). Copy number alterations of *JUNB* and *TWIST1* were found in only a minority of the SS patients (**Figure 1**).

Gene expression

GE analysis were performed on 55 SS, 19 EID and 4 HC cases in Leiden. Thirty samples were also analyzed in London with identical results in 28 samples (**Figure S2**), while two samples could not be analyzed due to a technical error.

DNM3, *TWIST1*, *EPHA4*, *PLS3* and *STAT4* were the most differentially expressed genes in SS patients compared to EID patients and healthy controls with 100% specificity ($P < 0.001$) (**Table 3**, **Figure S3**). Upregulation of *DNM3*, *TWIST1*, *EPHA4* and *PLS3* was found in 66–75% of SS patients, while *STAT4* was downregulated in 91% SS cases (**Figure 2**). Combining alterations in gene expression (*STAT4*, *TWIST1* and *DNM3* or *STAT4*, *TWIST1* and *PLS3*) we could distinguish 54 of 55 (98%) SS patients from all EID cases (sensitivity: 98%; specificity: 100%).

Aberrant gene expression of *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC* and *JUNB* was found in only a minority of the SS patients (**Table 3**).

DISCUSSION

In the present multicenter study, we investigate the diagnostic sensitivity and specificity of immunophenotypic and molecular biomarkers in SS using SOPs. We show that by using SOPs it is possible to obtain highly reproducible results for flow cytometry, CNV and GE analysis and demonstrate that loss of CD7 and CD26 by CD4+ T-cells, gain in copy number of *MYC* and loss of *MNT* as well as increased expression of *DNM3*, *TWIST1*, *EPHA4* and *PLS3* and decreased expression of *STAT4* are highly characteristic for Sézary cells.

In the current study the majority of SS cases have a CD4/CD8 ratio above 10 but we also demonstrate that a significant minority of cases (13%) does not fulfil this diagnostic criterion. In these cases additional immunophenotypic markers are clearly needed.

Comparison of results from previous flow cytometry studies is hindered by the use of different protocols and cell populations. In the present study we focused on CD4+ gated T-cells because CD4 is rarely lost by Sézary cells facilitating the comparison of expression levels of different immunophenotypic markers.

Previous studies reported that a CD4+CD26– cell population greater than 30% had a sensitivity of 97% and specificity of 100% in diagnosing peripheral blood involvement and suggested this cut off point as tentative diagnostic criterion for SS (Bernengo et al., 2001; Vonderheid and Bernengo, 2003). Indeed, loss of CD26 in more than 30% of CD4+ T-cells was found in 86% of SS cases but also in 53% of EID patients, resulting in a specificity of 47%. However, when using a percentage of 80% as cut off point, we found that 39 of 59 (66%) SS patients but none of the EID patients met this criterion.

These discrepant results can be explained by different flow cytometry protocols. We evaluated CD26 expression on CD4+ gated T-cells while Bernengo et al looked at CD4+CD26– cells on gated total lymphocytes. Indeed, when looking at the CD4+CD26– cells of 30% or more on total lymphocytes similar results were found (sensitivity: 80%; specificity: 95%, data not shown).

CD4+CD7– cells of more than 40% has also been suggested as tentative criterion in the diagnosis of SS (Vonderheid et al., 2002). Consistent with literature we found that loss of CD7 above 40% of the CD4+ T-cells is highly specific (100% specificity) but not a sensitive marker (sensitivity: 54%) (Harmon et al., 1996; Vonderheid and Bernengo, 2003; Nagler et al., 2012). Similar results were found for 40% or more CD4+CD7– cells on total lymphocytes (sensitivity: 42%; specificity: 100%, data not shown).

Flow cytometry results show that in 83% of Sézary patients CD4+ T-cells display loss of CD26 by more than 80% and/or loss of CD7 by more than 40% while this was never observed in EID patients. These observations are relevant as they can readily be included in immunophenotypic testing of erythrodermic patients.

Previous studies reported expression of killer cell immunoglobulin (KIR)-like receptor CD158k in 65–97% of SS patients (Poszepczynska-Guigne et al., 2004; Bahler et al., 2008; Klemke et al., 2008). Flow cytometry analysis, performed in both Leiden and Paris,

showed expression of CD158k in only 33% of SS cases (19 of 58 cases), and in the large majority of these Sézary patients (18 of 19) the CD158k antigen was expressed at low levels. This discrepancy can be explained by the fact that the present study was performed on frozen PBMCs instead of freshly isolated PBMCs. Indeed, a recent study on freshly isolated PBMCs from SS patients showed high CD158k expression on Sézary cells (Moins-Teisserenc et al., 2015).

For CNV and GE analysis the use of SOPs and custom made PCR platforms led to highly reproducible results as well. Gain of *MYC* and/ or loss of its antagonist *MNT* was found in 76% of Sézary patients but never in EID. Gain of *TWIST1* and *JUNB* was only detected in a small minority of SS patients.

In line with literature, we found upregulation of *DNM3*, *TWIST1*, *EPHA4* and *PLS3* and downregulation of *STAT4* in the majority of Sézary patients (Booken et al., 2008; Van Doorn R. et al., 2004; Goswami et al., 2012; Nebozhyn et al., 2006; Kari et al., 2003; Su et al., 2003). In contrast, only a minority of SS cases showed upregulation of *GATA3*, *CD1D*, *TRAIL*, *CDO1*, *JUNB* and *MYC* implying that these genes are not useful diagnostic markers. Why gain of *MYC* and loss of *MNT* which is observed in the majority of patients does not lead to upregulation of *MYC* expression is as yet unexplained.

Combined alterations in gene expression of *STAT4*, *TWIST1* and *DNM3* or *STAT4*, *TWIST1* and *PLS3* could distinguish 98% of SS patients from EID cases suggesting that this diagnostic panel will be useful as additional molecular criterion in the diagnostic differentiation between SS and EID.

In the present study 27 patients were diagnosed with SS prior to the inclusion of the study. However, no significant differences were found in the prevalence of the previously described markers between 27 patients already diagnosed with SS prior to inclusion of the study and 32 newly diagnosed SS patients at time of inclusion (**Table S1**). Similarly, the prevalence of

investigated markers was similar in 36 Sézary patients that received treatment during sample collection at inclusion and 23 SS patients that did not receive any form of treatment (**Table S2**). These observations argue that the observed immunophenotypic and molecular changes are stably expressed in Sézary cells.

In conclusion, we show that standardization of flow cytometry, CNV and GE procedures leads to strong reproducibility of results. We argue that in order to facilitate comparison of results from different centers it will be important to closely define the subset of cells that was investigated and based on the present study we suggest gating on CD4⁺ T-cells in future studies.

We propose that in those patients in whom distinction between SS and EID can still not be made using the current diagnostic criteria, these two additional diagnostic panels can be used:

1. loss of CD26 ($\geq 80\%$ CD4⁺ T-cells) and/ or loss of CD7 ($\geq 40\%$ CD4⁺ T-cells) for immunophenotypic analysis, and 2. combination of altered gene expression of *STAT4*, *TWIST1* and *DNM3* or *STAT4*, *TWIST1* and *PLS3* for molecular analysis.

MATERIALS & METHODS

Design of the study

To achieve sufficient power for the study a consortium of six EORTC centers with extensive experience in SS was formed including centers from Helsinki (Finland), London (England), Leiden (Netherlands), Mannheim (Germany), Turin (Italy) and Paris (France).

At time of inclusion peripheral blood samples were collected for investigation of: 1. expression of cell surface proteins by flow cytometry, 2. copy number variations (CNV) and 3. gene expression (GE) profiles. The markers were selected based on the literature and are presented in **Table S3**.

To optimize standardization and to prevent interdepartmental differences, standard operating procedures (SOPs) were produced for the workflow of: blood sampling, isolation of peripheral blood mononuclear cells (PBMCs) and enrichment for CD4⁺ T-cells (SOP 001), DNA isolation (SOP 002), RNA isolation (SOP 003), cDNA synthesis (SOP 004), copy number variation and gene expression assays (SOP 005), flow cytometry experiments (SOP 007) and the freezing and shipment of samples (SOP 008) (**Figure S4** and **supplementary file SOPs**). Much effort was put in standardizing flow cytometry analysis because this technique has been shown to have limited reproducibility in multicenter studies due to limited standardization of laboratory procedures, instrumental settings and interpretation of results (van Dongen et al., 2012; Westers et al., 2012).

To test if the use of SOPs leads to increased reproducibility, the flow cytometry experiments were performed in duplicate in Leiden and Paris on all samples and assays for CNV and GE were performed in Leiden and repeated in London for a selected number of cases.

In all participating centers the study was approved by their local institutional ethical review boards and written informed patient consent was obtained. Consensus meetings to compare experimental results were held on August 31, 2012 in Paris and October 31, 2013 in Leiden.

Patient selection and clinical characteristics

Between September 2009 and October 2013 a total of 103 patients were submitted with the following diagnosis: Sézary syndrome (n=72), erythrodermic inflammatory dermatoses (EID) (n=27) and healthy controls (HC) (n=4).

Inclusion criteria for the Sézary patients were: diagnosis of SS based on the recent WHO-EORTC criteria and available complete clinical data. Inclusion criteria for patients with EID were: presentation with erythroderma and blood results not meeting the SS blood criteria.

From the initial 72 patients with SS 13 were excluded, because of inferior sample quality (n=10) or insufficient clinical data (n=3). From the initial 27 EID patients eight were excluded, because of inferior sample quality (n=3) or insufficient clinical data (n=5).

The final study group consisted of 59 Sézary patients, 19 patients with EID and 4 HC. The SS group consisted of 32 patients with newly diagnosed SS and 27 patients with known SS.

Thirty-six Sézary patients received treatment at time of blood sampling (10 newly diagnosed SS and 26 with known SS). The treatment consisted of PUVA therapy (n=2), ECP as monotherapy or combined with immunomodulatory agents (n=12), prednisone alone or in combination with chlorambucil (n=9), monotherapy with interferon alfa, bexarotene, methotrexate or acitretin (n=11) and polychemotherapy (n=2).

The EID group included nine patients with atopic erythroderma, five patients with erythrodermic psoriasis, two patients with erythrodermic drug eruption, two patients with idiopathic erythroderma and one patient with paraneoplastic erythroderma secondary to a cholangiocarcinoma. None of the EID patients developed a lymphoma during follow-up (median follow-up 22 months, range 8-38).

Workup blood samples

PBMCs were isolated from peripheral blood and stored in liquid or vapour phase nitrogen. Part of the fresh PBMCs were enriched for CD4⁺ T helper cells, by depletion of non-CD4⁺ T-cells, resulting in > 95% purity for the CD4⁺ T-cell population, using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for CNV and GE assays. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at -20 °C.

RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. 2 µg RNA was reverse-transcribed in triplicate using the High Capacity cDNA

Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) using random priming in a final volume of 20 μ L. After synthesis, cDNA samples were stored at -20 °C. Detailed description of this workup of blood samples is found in supplementary file SOPs 001-004;008.

Flow cytometry

In Leiden and Paris flow cytometry was performed for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD27, CD45RA, CD45RO, CD158a, CD158b and CD158k, including isotype-specific controls (**Table S4, supplementary file SOPs 007**). Lymphocytes were gated from forward and sideward scatter patterns, secondly, antigen expression was assayed from CD4⁺ gated lymphocytes. Specific antigen expression was observed relative to auto fluorescence and (non-)specific signals obtained from each individual patient and a PBMC control sample. This control sample was derived from two healthy donors and functioned as internal reference sample each flow cytometry session. Samples were analyzed in a blinded setting.

Specific antigen expression in the population of gated cells (expression or loss) was displayed in percentages (**Figure 3a and b**). Antigen expression was considered “dim” if all gated cells showed diminished expression around the determined threshold (**Figure 3c**). For CD158a, CD158b and CD158k, expression below 5% of the gated cells was considered as no expression, but when intermediate expression of a single population of gated cells, surrounding a determined threshold, was found, this was characterised as low expressing antigen (for example CD158k^{low}) (**Figure 3d**).

Copy number variation assay

qPCR assays with FAM labelled hydrolysis MGB probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, *MNT* and reference genes *ABT1*, *ARG2* and *DNM3* (**Table S5**). Reference genes were selected from different large copy number-stable chromosomal regions in SS, selected from array based CGH experiments on 20 SS samples (Vermeer et al., 2008). Amplification efficiency was evaluated in triplicate, using eight 4-fold serial dilution points ranging from 3 ng/μL to 183 fg/μL DNA concentration, under optimised primer and hydrolysis probe concentrations. Assays with amplification efficiency value (E) between 90 and 100% and correlation coefficient (R^2) above 0.98 were accepted for CNV analysis. Assays were performed on custom made PCR plates (Life Technologies) following supplementary file SOP 005.

Data was normalised against reference genes and relative to the CR using the $\Delta\Delta C_q$ method and is presented as relative copy number, where 2 stands for diploid DNA (Livak and Schmittgen, 2001). The following thresholds were maintained for the CNV data: 1.5-2.5 was considered as diploid (normal) DNA, > 2.5 as gain in copy number and < 1.5 as loss.

Gene expression assay

GE qPCR assays with FAM labelled hydrolysis MGB probes (Life Technologies) were developed and validated, as described for CNV qPCR assays, for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, *STAT4* and reference genes *ARF5*, *ERCC3* and *TMEM87A* (**Table S5**). Stably expressed reference genes were selected from microarray experiments on SS samples and validated in SS and EID samples according to the GeNorm method (Vandesompele et al., 2002; Van Doorn R. et al., 2004; Booken et al., 2008). Assays were performed on custom made PCR plates (Life Technologies) following SOP 005. ROC curve analysis were used to determine fixed cut-off thresholds for each individual gene expression qPCR assay with a specificity of 100% and an accuracy above

0.80. A one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant.

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Tables:

Table 1. Clinical characteristics at diagnosis of the 59 SS and 19 EID patients.

Values at diagnosis SS versus erythroderma EID	SS n=59	EID n=19
Male-female ratio	37:22	16:3
Age, median (range), y	65 (32-89)	67 (29-86)
Erythroderma	46/52 (88%)	19/19 (100%)
Pruritus	45/52 (87%)	13/19 (68%)
Ectropion	6/52 (12%)	1/19 (5%)
Hyperkeratosis hand/feet	21/52 (40%)	3/19 (16%)
Palpable lymphadenopathy	21/52 (40%)	4/19 (21%)
Lymphadenopathy confirmed by CT scan	17/41 (41%)	1/7 (14%)
Leukocytes $\geq 10.0 \times 10^9/L$	40/56 (71%)	5/14 (36%)
CD4/CD8 ratio ≥ 10.0	53/57 (93%)	1/12 (8%)
Absolute Sézary cell count $\geq 1000 \text{ mm}^3$	34/43 (79%)	0/10 (0%)
T-cell clone in peripheral blood	59/59 (100%)	1/17 (6%)
Identical T-cell clone in blood and skin	32/38 (84%)	0/3 (0%)

Table 2. Overview of the tested flow cytometry markers in 59 SS and 19 EID patients at inclusion of the study.

Markers for SS described in literature	SS n=59	EID n=19	Sensitivity	Specificity
CD4/CD8 ratio ≥ 10	46/53	1/12	87%	92%
CD4+CD7- $\geq 40\%$	32/59 ¹	0/19	54%	100%
CD4+CD26- $\geq 30\%$	51/59 ²	10/19	86%	47%
CD158a*	2/58	0/19	3%	100%
CD158b*	13/59	1/19	22%	95%
CD158k*	19/58	1/19	33%	95%

*Low expression and expression of 5% or more of the CD4+ lymphocytes.

¹Including 4/7 SS patients with a CD4/CD8 ratio below 10.

²Including 5/7 SS patients with a CD4/CD8 ratio below 10.

Table 3. Results of aberrant gene expression in all tested genes in 55 SS relative to 19 EID and 4 HC. With the ROC curve analysis a threshold was established at a specificity of 100% and an accuracy above 0.80. *PLS3*, *DNM3*, *TWIST1*, *EPHA4* and *STAT4* and were found to be useful diagnostic markers in SS.

Up-/down regulation	SS (n=55)	Sensitivity
<i>PLS3</i>	36/55	66%
<i>DNM3</i>	41/55	75%
<i>CDO1</i>	20/55	36%
<i>TRAIL</i>	4/55	7%
<i>CD1D</i>	6/55	11%
<i>GATA3</i>	2/55	4%
<i>MYC</i>	0/55	0%
<i>JUNB</i>	9/55	16%
<i>TWIST1</i>	38/55	69%
<i>EPHA4</i>	36/55	66%
<i>STAT4</i>	50/55	91%

*Figure legends:***Figure 1. Copy number variation results for *JUNB*, *TWIST1*, *MNT* and *MYC*.**

The gains and losses in copy number in 58 SS compared to 17 EID cases are shown as normalized relative copy number, where 2 represents diploid DNA. The dotted lines signifies the chosen thresholds for gain and loss, 2.5 and 1.5, respectively.

Figure 2. Gene expression results for *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4*.

The differential gene expression is shown as relative normalized mRNA levels in 55 SS compared to 19 EID and 4 HC cases. **** represents the statistical significant difference in gene expression in SS compared to EID and HC ($P < 0.001$). The dotted lines represent the thresholds for differential expression, determined with ROC curves with a specificity of 100%. The Y-axis represents the relative mRNA expression with varying scale in all figures.

Figure 3. Interpretation of flow cytometry results.

a. A single peak located on the right side of the determined threshold represents a single population with positive staining for CD7 in 97% of the gated cells; **b.** Two distinct populations of the gated cells, in which 47% of the cells show positive expression for CD7 and 53% show negative staining; **c.** Diminished expression for CD7 of a single population of gated cells, surrounding a determined threshold, is indicated as “dim”. **d.** The specific CD158k antigen expression signal (indicated in red) is slightly shifted to the right compared to its auto fluorescence and isotype control signals (green and blue lines), as is indicated in yellow. This implicates that the gated cells do express CD158k but at very low level, indicated as “low”.





